

Inhibition of Transpeptidase Activity in *Escherichia coli* by Thienamycin

BRIAN A. MOORE,* SIDNEY JEVONS, AND KEITH W. BRAMMER

Pfizer Central Research, Sandwich, Kent, England

Received for publication 15 March 1979

Thienamycin was shown to be a more potent inhibitor than ampicillin of the enzyme peptidoglycan transpeptidase from *Escherichia coli*.

β -Lactam antibiotics kill bacteria by inhibiting reactions involved in the final stages of cell wall biosynthesis (1, 2). It is generally believed that a membrane-bound transpeptidase, which catalyzes the cross-linking of neighboring amino acid side chains during cell wall peptidoglycan synthesis, is a major target for β -lactam action and that inhibition of this enzyme leads to cell death (15, 16). Furthermore, all β -lactams antibiotics so far investigated have been shown to bind irreversibly to one or more of six penicillin-binding proteins (PBPs) found in the cytoplasmic membrane of *Escherichia coli* (10). Although the roles of all six PBPs have not been fully elucidated, it is assumed that one or more are the targets for the lethal action of penicillins and cephalosporins (10).

Thienamycin is a novel, broad-spectrum, highly potent β -lactam antibiotic obtained from cultures of *Streptomyces cattleya* (14), and it is known to bind with high affinity to all six *E. coli* PBPs (11). It is believed that a component of PBP 1 has the properties expected of the enzyme peptidoglycan transpeptidase (11, 13). Thienamycin binds to PBP 1 with higher affinity than does almost any other β -lactam antibiotic so far studied (10, 11). However, no direct evidence that thienamycin actually inhibits the transpeptidase has so far been reported. We have produced evidence demonstrating transpeptidase inhibition by thienamycin and have shown that the potency of thienamycin as a transpeptidase inhibitor is commensurate with its antibacterial activity.

The transpeptidase assay system used was that of Mirelman et al. (6), modified by replacement of the pH 8.3 tris(hydroxymethyl)aminomethane buffer with a pH 7.0 potassium phosphate buffer (10 mM final concentration in the assay mixture). This modification was necessary due to the instability of thienamycin at pH 8.3 (J. S. Kahan et al., U.S. Patent 3,950,357, April 1976).

The transpeptidase assay system of Mirelman et al. uses *E. coli* cells made permeable to ex-

ogenous nucleotide-sugar peptidoglycan precursors by ether treatment, producing so-called ether-treated bacteria (ETB). The exogenous precursors used are uridine-5'-diphospho-*N*-acetyl-D-[U - 14 C]-glucosamine (UDP-[14 C]-GlnNAc) and uridine-5'-diphospho-*N*-acetylmuramyl-L-alanyl-D-glutamyl-meso-diaminopimelyl-D-alanyl-D-alanine (UDP-MurN-pentapeptide). Simultaneous incorporation of these by ETB proceeds via macromolecular peptidoglycan precursors which are then cross-linked to preexisting cell wall exclusively by penicillin-sensitive transpeptidation (6). Transpeptidase activity is measured by the incorporation of radioactivity into sodium dodecyl sulfate-insoluble, cross-linked peptidoglycan. We have used the method described by Mirelman et al. to prepare ETB (in 10 mM phosphate buffer, pH 7.0) from *E. coli* 146, a penicillin-sensitive clinical isolate. The protein content of ETB was measured by the method of Lowry et al. (4). The UDP-MurNAc-pentapeptide substrate was purified by gel filtration (Sephadex G10) and ion-exchange chromatography (Dowex-1-chloride) from trichloroacetic acid-soluble extracts of vancomycin-treated *Bacillus cereus* ATCC 11778, according to the method of Lugtenberg et al. (5). The final concentration of purified material was determined by *N*-acetyl amino sugar estimation (12).

Transpeptidase activity and its inhibition in *E. coli* 146 ETB were determined as follows. ETB (0.2 mg of protein) were incubated in the presence or absence of antibiotic for 1 h at 37°C in 10 mM phosphate buffer (pH 7.0) containing 50 mM NH_4Cl , 20 mM MgCl_2 , 0.25 mM di-sodium adenosine 5'-triphosphate and 0.5 mM 2-mercaptoethanol in a final volume of 200 μl . Incubation mixtures also contained the substrates UDP[14 C]-GlnNAc (40 μmol ; 15.8 mCi/mmol) and unlabeled UDP-MurNAc-pentapeptide (50 μmol). Reactions were terminated with 1 ml of 4% sodium dodecyl sulfate, and the mixtures were boiled for 30 min. Insoluble material was collected on 0.45- μm membrane filters (Millipore Corp.) and washed with 7 volumes of

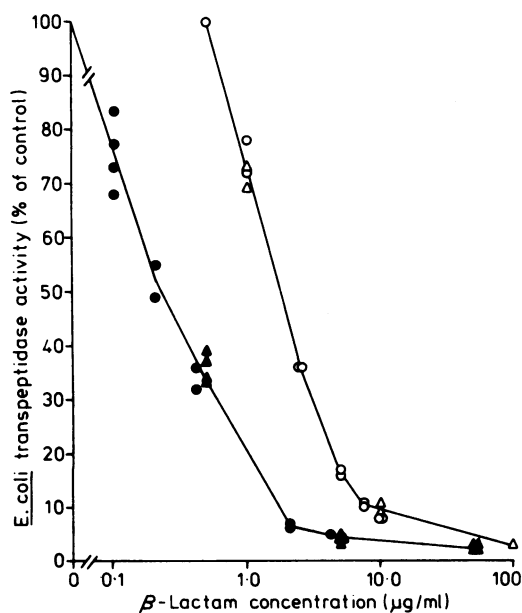


FIG. 1. Transpeptidase activity in ETB derived from *E. coli* 146 was measured as described in the text. The uninhibited (control) activity is expressed as 100%, and the effects of various concentrations of ampicillin (O, Δ) and thienamycin (\bullet , \blacktriangle) upon this activity are shown. The symbols indicate that two separate experiments were performed.

distilled water. The filters were then dried, and the associated radioactivity was determined by scintillation counting in Instagel scintillation fluid (Packard) with a Nuclear Chicago Mark I liquid scintillation counter.

The effects of various concentrations of ampicillin (a known transpeptidase inhibitor[3]) and thienamycin on the transpeptidase reaction were determined and expressed as a percentage of the control reaction (Fig. 1). No significant inhibition occurred at ampicillin concentrations below 0.5 $\mu\text{g/ml}$ and thienamycin concentrations below 0.05 $\mu\text{g/ml}$. It is clear from Fig. 1 that thienamycin is a potent transpeptidase inhibitor, having a 50% inhibitory concentration of 0.23 $\mu\text{g/ml}$ compared with 1.8 $\mu\text{g/ml}$ for ampicillin, showing thienamycin to be about eight times more potent than ampicillin.

Minimal inhibitory concentrations of thienamycin and ampicillin against *E. coli* 146 were also determined by using an agar dilution technique. *E. coli* 146 was grown in Iso-Sensitest broth (Oxoid Ltd.) at 37°C for 18 h. The culture was then diluted 100-fold in broth and inoculated onto Iso-Sensitest agar (Oxoid Ltd.) containing serial doubling dilutions of antibiotic. Inoculation was performed with an automatic inoculator (Denley Instruments), giving an in-

oculum of approximately 10^4 colony-forming units. After incubation at 37°C for 18 h the minimal inhibitory concentration was recorded as the lowest concentration of antibiotic which completely inhibited visible growth of the organism. Both ampicillin and thienamycin inhibited the growth of *E. coli* 146, with minimal inhibitory concentrations 3.1 and 0.31 $\mu\text{g/ml}$, respectively. Thienamycin was thus about 10 times more potent antibacterially than ampicillin. This difference is consistent with the relative potencies against the transpeptidase enzyme. (It is recognized that the sensitivity of target enzymes is not the sole determinant of the susceptibility of intact organisms to inhibition by β -lactam antibiotics and that other factors, such as the bacterial outer membrane and the presence of β -lactamase enzymes, may play an important role [7, 8]).

It is reasonable to suppose that the enzyme peptidoglycan transpeptidase is a target for thienamycin action, as it is for other β -lactam antibiotics (2, 15, 16). This is not necessarily the only important target site, however. For example, there is evidence that thienamycin binds with highest affinity to PBP 2 (11), which is not the transpeptidase, and that reduction of this affinity alone, by mutation, leads to a decrease in susceptibility of the organism to thienamycin (9). No enzyme activity has yet been associated with PBP 2.

In *E. coli*, a component of PBP 1 has the properties which might be expected of the enzyme peptidoglycan transpeptidase (11, 13). Thienamycin binds strongly to PBP 1, and the results of our experiments indicate that thienamycin strongly inhibits the transpeptidase enzyme.

ACKNOWLEDGMENTS

We thank Judy Pegg and Keith Holmes for excellent technical assistance. We also thank Merck, Sharp & Dohme for the gift of thienamycin.

LITERATURE CITED

1. Blumberg, P. M., and J. L. Strominger. 1974. Interaction of penicillin with the bacterial cell: penicillin-binding proteins and penicillin-sensitive enzymes. *Bacteriol. Rev.* 38:291-335.
2. Ghuyssen, J.-M. 1977. The concept of the penicillin target from 1965 until today. *J. Gen. Microbiol.* 101:13-33.
3. Izaki, K., M. Matsushashi, and J. L. Strominger. 1968. Biosynthesis of the peptidoglycan of bacterial cell walls. XIII. Peptidoglycan transpeptidase and D-alanine carboxypeptidase: Penicillin-sensitive enzymatic reactions in strains of *Escherichia coli*. *J. Biol. Chem.* 243:3180-3192.
4. Lowry, O. H., N. J. Rosebrough, A. L. Farr, and R. J. Randall. 1951. Protein measurement with the Folin phenol reagent. *J. Biol. Chem.* 192:265-275.
5. Lugtenberg, E. J. J., A. van Schijndel-Van Dam, and T. H. M. van Belleghem. 1971. In vivo and in vitro action of new antibiotics interfering with the utilization

- of *N*-acetyl-glucosamine-*N*-acetyl-muramyl-pentapeptide. *J. Bacteriol.* **108**:20-29.
6. Mirelman, D., Y. Yashouv-Gan, and U. Schwarz. 1976. Peptidoglycan biosynthesis in a thermosensitive division mutant of *Escherichia coli*. *Biochemistry* **15**: 1781-1790.
 7. Richmond, M. H., and N. A. C. Curtis. 1974. The interplay of β -lactamases and intrinsic factors in the resistance of gram-negative bacteria to penicillins and cephalosporins. *Ann. N. Y. Acad. Sci.* **235**:553-567.
 8. Richmond, M. H., and R. B. Sykes. 1973. The β -lactamases of gram-negative bacteria and their possible physiological role. *Microb. Physiol.* **9**:31-88.
 9. Spratt, B. G. 1978. *Escherichia coli* resistance to β -lactam antibiotics through a decrease in the affinity of a target for lethality. *Nature (London)* **274**:713-715.
 10. Spratt, B. G. 1977. Properties of the penicillin-binding proteins of *Escherichia coli* K12. *Eur. J. Biochem.* **72**: 341-352.
 11. Spratt, B. G., V. Jobanputra, and W. Zimmermann. 1977. Binding of thienamycin and clavulanic acid to the penicillin-binding proteins of *Escherichia coli* K12. *Antimicrob. Agents Chemother.* **12**:406-409.
 12. Strominger, J. L. 1957. Microbial uridine-5'-pyrophosphate *N*-acetyl amino sugar compounds. I. Biology of the penicillin-induced accumulation. *J. Biol. Chem.* **224**:509-523.
 13. Suzuki, H., Y. Nishimura, and Y. Hirota. 1978. On the process of cellular division in *Escherichia coli*: a series of mutants of *E. coli* altered in the penicillin-binding proteins. *Proc. Natl. Acad. Sci. U.S.A.* **75**:664-668.
 14. Tally, F. P., N. V. Jacobus, and S. L. Gorbach. 1978. *In vitro* activity of thienamycin. *Antimicrob. Agents Chemother.* **14**:436-438.
 15. Tipper, D. J., and J. L. Strominger. 1965. Mechanisms of action of penicillins: a proposal based on their structural similarity to acyl-D-alanyl-alanine. *Proc. Natl. Acad. Sci. U.S.A.* **54**:1133-1141.
 16. Wise, E. M., and J. T. Park. 1965. Penicillin: its basic site of action as an inhibitor of a peptide cross-linking reaction in cell wall mucopeptide synthesis. *Proc. Natl. Acad. Sci. U.S.A.* **54**:75-81.